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We are working to develop a novel genomic subtraction method to rapidly identify all chromosomal regions that undergo Loss of Heterozygosity (LOH) in breast cancer. LOH is a major mechanism in the genomic alteration that transforms a normal cell into an unregulated tumor cell. The identification of all regions of LOH will identify causative genes and gene interactions and will yield new understanding of the etiology and pathogenesis of breast cancer and lead to new approaches to detection and treatment.

We have designed several genomic subtraction methods that should isolate enriched populations of DNA fragments representing LOH regions in individual breast cancer cell lines. Our approach exploits the minor degree of DNA sequence heterogeneity expected between the members of each chromosome pair. Genomic subtraction requires presence/absence differences, and we transform the DNAs to create such differences. Subtraction of tumor DNA from normal tissue DNA from the same individual should leave a remainder population of DNA fragments enriched for the different sequences missing from the tumor DNA due to LOH. We are currently refining and comparing alternative methods in model systems to determine the superior approach.

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INTRODUCTION

Our goal is to use genomic subtraction to identify chromosomal regions of Loss of Heterozygosity (LOH) in breast cancer cells. Patterns of LOH will reveal new information about progressive genetic changes in breast cancer, enhancing our understanding of this disease and offering new opportunities for therapeutic intervention.

LOH is a frequent and characteristic event in many or all tumor types, and it operates to unmask deleterious mutations that have occurred or been inherited in particular genes that control growth and differentiation. When the normal allele is lost, only the altered function is left. Thus LOH is an integral part of the process of deregulating a cancer cell and may be crucial in the development of most tumors. LOH clearly is important in the progression of malignant disease. Losses at 1p and 11p15 have been associated with metastasis of breast cancer to regional lymph nodes, and LOH at 1q was associated with early disease recurrence. Thus LOH patterns can yield information regarding clinical properties of tumors as well as pointing to important oncogenes involved in their genesis. Any LOH that is observed repeatedly probably points to a gene whose alteration is important in malignant progression. Identification of the altered gene and its function would offer new opportunity for diagnosis, intervention and therapy. A systematic study of LOH over the whole genome in many individuals at various stages has not been done for breast cancer or any other tumor. A feasible approach to this goal will bring promise of much valuable new information. We are developing a genomic subtraction method that derives informative probes from the individual's own DNA. We will use these as probes for Fluorescent in situ Hybridization (FISH) to identify all areas of LOH over the entire genome in a single analysis.

Our strategy exploits the fact that tumor DNA is virtually identical to normal DNA even though there may be chromosome rearrangements or amplifications. Only deletions and other regions of LOH are changes where allelic sequences are missing. Genomic subtraction selects DNA specifically from these regions of difference. Our subtraction strategy exploits small allelic sequence differences and captures sequences that are nearly identical between the retained and the "lost" allele in tumor genomes undergoing LOH. In the case of LOH in tumors only the alleles from one parent are missing; the homologous sequences from the other parent are present, usually in two copies. There is no presence/absence (+/-) difference that would leave a remainder after subtraction of one genome from the other. The system can be transformed, however, into one with +/- differences suitable for genomic subtraction, and we use DOP-PCR to accomplish this.

We create sampled populations of sequences for subtraction by DOP-PCR, or Degenerate Oligonucleotide Primed PCR, which uses a short primer sequence corresponding to a commonly occurring hexamer to amplify intervening DNA. Allelic differences in whether or not a DOP priming site is present will result in the presence or absence of sequences from the amplified populations. These differences are present at high frequency. Subtraction of the amplified tumor DNA from amplified normal DNA

from the same individual yields specifically those sequences that differ between alleles in the region(s) of LOH.

Advantages inherent in the subtractive approach are that it does not depend on previously defined RFLP or SSRP markers that may or may not be informative in each individual; rather, it exploits whatever uncharacterized DNA sequence differences there are in each individual. It produces a single comprehensive analysis of LOH across the entire genome simultaneously, rather than requiring an intensive series of individual marker assays. FISH painting with subtracted DNAs from different individuals will identify consistent sites of LOH, and the staining patterns will define the "minimum region of overlap" of LOH in different tumors; this will guide positional cloning and identification of novel oncogenes and tumor suppressor genes.

BODY

Our objectives for the first year were the development of the methods and the demonstration of genomic subtraction using simple models. We proposed to begin by demonstrating genomic subtraction in a YAC-Yeast system and later in the more complex congenic mouse DNAs and tumor cell line DNAs. Genomic subtraction technology is very challenging, and the yeast system still provides the appropriate platform for the development of these techniques.

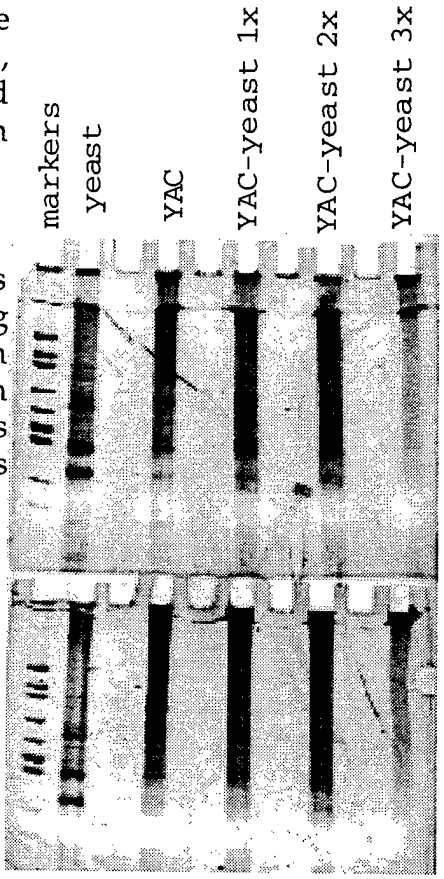
In developing the genomic subtraction technique we have investigated two critical variables, the genome sampling strategy and the subtraction method. In sampling the genome we aim for a simple and robust technique that produces a very broad representation of the genome. The subtraction method should also be simple and robust, but the primary aim is effectiveness, i.e., subtraction should be as near to 100% as can be achieved.

We compared three sampling strategies, degenerate-oligo-primed PCR (DOP-PCR), inter-repeat-sequence PCR (IRS-PCR), and restriction fragment length polymorphisms (RFLP). We also compared three methods of subtraction, physical subtraction through phenol extraction of avidin-bound biotin-derivatized subtractor DNA, solid phase subtraction with avidin-beads, and suicide subtraction through enzymatic degradation of subtractor DNA and its hybrids.

Our initial experiments used mouse DNAs, RFLP sampling and phenol subtraction. The products were cloned as plasmid libraries and analyzed by hybridization. The RFLP sampling method -- size fractionating restricted DNAs by agar gel electrophoresis, slicing the gel and extracting the DNA fractions -- proved feasible but quite cumbersome in repeated use. When we analyzed the cloned subtraction products most carried repetitive DNA, and no difference was apparent between the starting DNA and the subtracted DNA. This strategy had no means to amplify the subtracted material so that it could be subjected to additional rounds of subtraction to improve the enrichment. It is necessary to incorporate PCR amplification of the recovered subtraction products to allow further subtraction and to increase the yield of the final product for use as probes.

We then compared the two PCR methods for genome sampling and found that the DOP-PCR yielded a much broader and more useful sampling, as well as being very straightforward and reproducible. All subsequent development has employed this sampling method. The generation of the initial genome sample by PCR then allows additional amplification as needed using the same PCR primers.

We have further compared in a number of experiments alternative methods of biotinylating the subtractor DNA and physically removing the avidin-bound biotin-DNA. We have used the photobiotinylation method predominately, but have also used biotin-derivatized oligonucleotide primers in the PCR amplification of the subtractor DNA. Both methods appear to work without a clear advantage for either. We also compared the phenol-chloroform extraction of the avidin-biotin-DNA complex to binding the biotin-DNA with avidin conjugated to magnetic beads. In these experiments we also incorporated tritium tracer in the subtractor PCR product, and this revealed that a very small (1-3%) fraction of the subtractor DNA was resistant to removal, perhaps due to incomplete biotinylation. Since the subtractor/target DNA ratio is at least 30/1, this is a serious problem. This is confirmed by analysis of the remainder after subtraction; even after three rounds most of the YAC-yeast products are still yeast sequences. Nonetheless, subtraction does remove major yeast components and alter the composition of the YAC DNA sample as shown in the figure.



This figure shows duplicate polyacrylamide gels comparing DOP-PCR products of yeast, YAC (including the yeast host), and the products of 3 rounds of subtraction of yeast from YAC. With increasing rounds of subtraction the prominent discrete bands in the yeast and YAC lanes appear to be removed leaving a uniform smear of species in the final lane.

We have taken several approaches to address the problem of residual subtractor DNA besides the straightforward one of refining and improving the degree of biotinylation of the subtractor. We have developed a "suicide subtractor" approach in which the subtractor and any target strands hybridized to it are degraded and destroyed. To do this we incorporate uridine into the subtractor PCR product in place of thymidine. This does not alter the base pairing specificity of the subtractor which hybridizes to the

target strands to be subtracted. After hybridization, treatment with Uracil-DNA Glycosylase (UDG) removes the uracil bases from the phosphodiester backbone of the DNA strand. These abasic sites are then sensitive to Nuclease S1 cleavage which nicks the abasic strand and then cleaves the strand opposite the nick, thereby degrading both the subtractor strand and the target strand. Initial experiments with only 1 round of subtraction show evidence of subtraction but are not sufficient to quantify enrichment and will require additional rounds of subtraction. This approach seems quite promising.

A related tactic uses a cleavable DOP primer to prepare the subtractor DNA. After PCR preparation of the subtractor the primer sequences on the ends are cleaved off, rendering them inert in future rounds of PCR. To also make the target strands that are hybridized to the subtractor strands incapable of being amplified, the primer ends are removed from the heteroduplexes after PERT hybridization by "polishing" the ends with Mung Bean Nuclease to remove single strand overhangs. Thus the target strands will lose their priming sites for further amplification and only the target homoduplexes will retain priming sites and reamplify. The cleavable DOP primer is shown:

```
TAATACGACTCACTATAGGGatccNNATGTGG
[------ T7 --------]     [-DOP--]
                   [Bam-]
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After amplification of the subtractor DNA the T7 portion of the primer is cleaved off with BamHI. Any rounds of PCR after the initial genome sampling use only the 20-mer T7 primer, ensuring that the subtractor is inert to amplification. We will pursue this strategy in addition to the others above to bring to successful practice the subtraction technology we need for this project.

CONCLUSIONS

This is a challenging project that presents many problems. We have made progress in developing a successful genomic subtraction strategy and have described additional approaches that should bring this project to a successful conclusion and provide novel and important characterizations of the patterns of Loss of Heterozygosity that reflect the initiation and progression of breast cancer.